

# S-nitrosylated proteins of a medicinal CAM plant *Kalanchoe pinnata* – ribulose-1,5-bisphosphate carboxylase/oxygenase activity targeted for inhibition

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## Keywords

biotin switch technique; *Kalanchoe pinnata*;  
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Nitric oxide (NO) is a signaling molecule that affects a myriad of processes in plants. However, the mechanistic details are limited. NO post-translationally modifies proteins by S-nitrosylation of cysteines. The soluble S-nitrosoproteome of a medicinal, crassulacean acid metabolism (CAM) plant, *Kalanchoe pinnata*, was purified using the biotin switch technique. Nineteen targets were identified by MALDI-TOF mass spectrometry, including proteins associated with carbon, nitrogen and sulfur metabolism, the cytoskeleton, stress and photosynthesis. Some were similar to those previously identified in *Arabidopsis thaliana*, but kinesin-like protein, glycolate oxidase, putative UDP glucose 4-epimerase and putative DNA topoisomerase II had not been identified as targets previously for any organism. *In vitro* and *in vivo* nitrosylation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), one of the targets, was confirmed by immunoblotting. Rubisco plays a central role in photosynthesis, and the effect of S-nitrosylation on its enzymatic activity was determined using NaH<sup>14</sup>CO<sub>3</sub>. The NO-releasing compound S-nitrosoglutathione inhibited its activity in a dose-dependent manner suggesting Rubisco inactivation by nitrosylation for the first time.

Nitric oxide (NO), a water- and lipid-soluble gaseous free radical, has emerged as a key signaling molecule in plants. Pharmacological investigations using NO donors and inhibitors have implicated NO in diverse processes, from seed germination to cell death [1–3]. However, information about the NO-mediated signal transduction pathway(s) or the components involved is limited. An important biological role of NO may involve post-translational modification of proteins by: (i) S-nitrosylation of thiol groups, (ii) nitration of tyrosine and tryptophan (biological nitration), (iii) oxidation of thiols and tyrosine, and (iv) binding to

metal centers [4]. S-nitrosylation of cysteine residues in the target protein is a principle and reversible modification by NO mediating its cyclic guanosine monophosphate (cGMP)-independent effects [5].

NO nitrosylates transition metals, whereas NO-derived species such as NO<sub>2</sub>, N<sub>2</sub>O<sub>3</sub> and transition metal–NO adducts nitrosylate cysteine residues in proteins. Low-molecular-weight nitrosothiols such as S-nitrosoglutathione (GSNO) nitrosylate target proteins via transnitrosation, which involves direct transfer of a NO group [6]. S-nitrosylation further promotes disulfide bond formation in the neighboring

## Abbreviations

Biotin-HPDP, N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide; CAM, crassulacean acid metabolism; GSH, glutathione; GSNO, S-nitrosoglutathione; MMTS, methyl methanethiosulfonate; NEM, N-ethylmaleimide; NO, nitric oxide; PEG, polyethylene glycol; PEPC, phosphoenolpyruvate carboxylase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SNP, sodium nitroprusside.

thiols, thereby affecting protein activity [5]. It appears that the 3D microenvironment of the reactive thiol may in fact enhance the nitrosative reactivity [7].

In *Arabidopsis thaliana*, genomic and proteomic approaches have identified NO-responsive transcripts and proteins [8,9]. Microarray analysis has indicated that 2% of the transcripts in the *Arabidopsis* genome are NO-responsive. Analysis of sodium nitroprusside (SNP)-treated seedlings showed 342 up- and 80 down-regulated genes, encoding disease resistance proteins, transcription factors, redox proteins, ABC transporters, signaling components, and enzymes involved in hormone (ethylene and methyl jasmonate) biosynthesis and secondary metabolism [8]. A proteomics approach, using the biotin switch technique, identified 63 S-nitrosylated proteins from cell cultures and 52 such proteins from leaves, including stress-related, redox-related, signaling/regulatory, cytoskeletal and metabolic proteins [9].

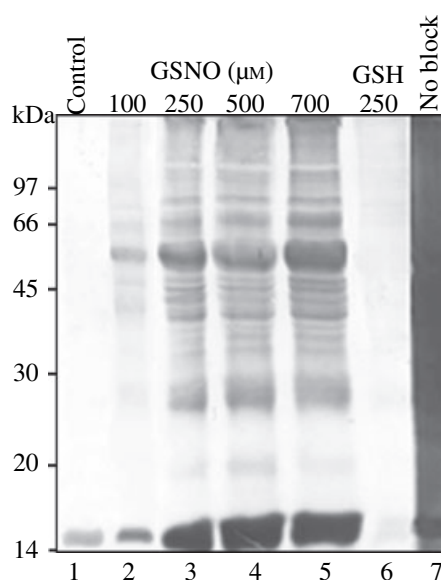
Compared to *Arabidopsis*, a C<sub>3</sub> plant, little is known about S-nitrosylation in crassulacean acid metabolism (CAM) plants. Our studies focus on a CAM plant, *Kalanchoe pinnata*, which belongs to the Crassulaceae family and possesses numerous medicinal properties, including antibacterial, anti-allergic, antihistamine, analgesic, anti-ulcerous, gastroprotective, immunosuppressive, sedative, antilithic and diuretic [10]. The understanding of how the plant or its extracts control such a diverse set of processes is in its infancy, and ascertaining the mechanisms for each medicinal property is a huge task. Therefore, we are interested in signaling molecules that are known to have global and multiple effects, such as NO, with respect to their possible involvement in the biology of CAM plants such as *K. pinnata*.

We report here the identity of the major S-nitrosylated proteins of *K. pinnata*, show that ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a S-nitrosylated target, and demonstrate that Rubisco enzyme activity is inhibited upon nitrosylation.

## Results

### Detection of S-nitrosylated proteins in *K. pinnata*

GSNO treatment readily nitrosylated several soluble proteins from *K. pinnata* (Fig. 1), but its inactive analog, glutathione (GSH, 250  $\mu$ M), did not nitrosylate any proteins (Fig. 1, lane GSH). Thus, protein nitrosylation by GSNO seems specific. An abundant 16 kDa polypeptide was among the nitrosylated proteins detected, and its nitrosylation increased with increasing GSNO concentrations, becoming saturated between 500–

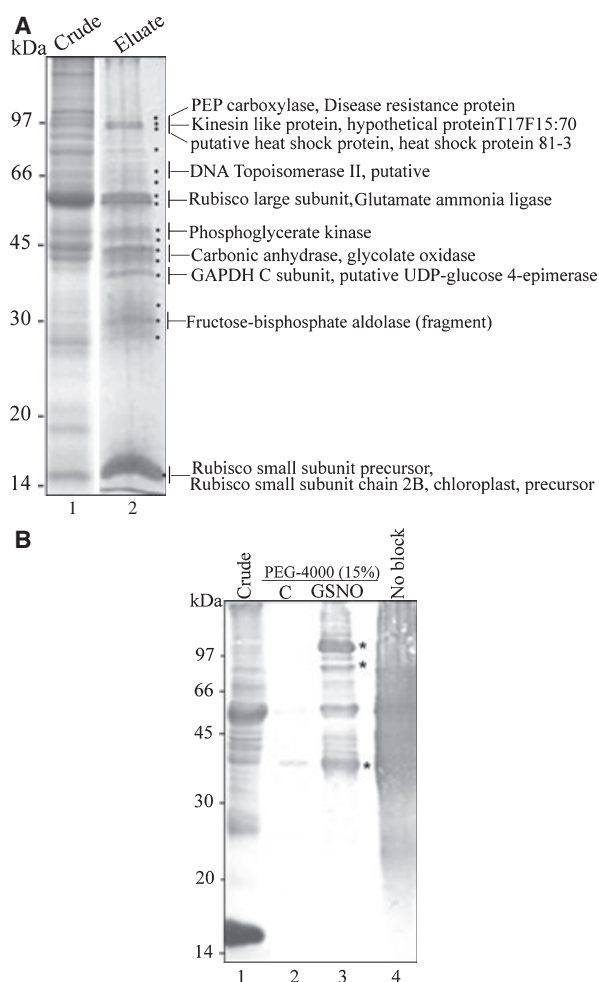


**Fig. 1.** Immunoblot of S-nitrosylated proteins from *Kalanchoe pinnata* leaf using the biotin switch technique. Protein extracts (240  $\mu$ g) were used either as such (lane 1) or treated with the indicated concentrations of GSNO (lanes 2–5) or GSH (250  $\mu$ M, lane 6) for 20 min. Lane 7 represents the unblocked sample; the other samples were blocked with NEM (50 mM). After biotinylation, proteins were separated by 12% SDS–PAGE, transferred to nitrocellulose membrane and then probed with anti-biotin IgG (1 : 500 dilution).

700  $\mu$ M. Addition of *N*-ethylmaleimide (NEM) inhibited nitrosylation of all proteins except the 16 kDa polypeptide, which retained a residual level that was maintained even at a 10-fold higher concentration of NEM (supplementary Fig. S1). Omitting biotin from the assay did not yield any signal, suggesting that the polypeptide is not endogenously biotinylated. Treatment with dithiothreitol (a thiol-specific reductant) reversed the protein S-nitrosylation.

### Purification and identification of S-nitrosylated proteins by biotin–avidin affinity chromatography

To identify the S-nitrosylated proteins, neutravidin affinity chromatography was used to purify biotinylated proteins from *K. pinnata* leaf extracts as described in Experimental procedures. The crude fraction and the purified protein eluates were resolved by SDS–PAGE and silver-stained to visualize the polypeptides. Eighteen polypeptides, ranging in size from 116 to 29 kDa, were resolved (Fig. 2A, eluate). Most of the enriched, stained proteins electrophoresed at or above 28 kDa, except two polypeptides between 14 and 16 kDa. Polyethylene glycol (PEG) fractionation was used to reduce the abundance of Rubisco in the cell extracts to ascertain that the highly nitrosylated



**Fig. 2.** Purification and fractionation of the nitrosylated proteins used for identification. (A) Silver-stained SDS–polyacrylamide gel (12%) showing the profile of neutravidin–agarose-purified S-nitrosylated proteins from *Kalanchoe pinnata*. Leaf proteins (5 mg) were treated with GSNO (250  $\mu$ M) and biotinylated using the biotin-switch technique. Lane 1, crude extract; lane 2, purified fraction of S-nitrosylated proteins. Molecular mass markers (kDa) are shown on the left. Dots indicate the positions of polypeptides excised from the gel for trypsinization and MALDI-TOF mass spectrometry analysis. The names of the identified proteins are listed next to their electrophoretic position. (B) Immunoblot of the PEG-4000-precipitated fraction of *K. pinnata* leaf extracts. Supernatant (240  $\mu$ g protein) collected after 15% PEG-4000 precipitation of the extracts was treated with (lane 3, GSNO) or without (lane 2, C) 250  $\mu$ M GSNO and then subjected to the biotin switch technique. Lane 1 is a GSNO-treated sample prior to PEG-4000 precipitation and lane 4 is the unblocked sample. Blots were probed with anti-biotin IgG. Asterisks next to the protein bands indicate the targets that were revealed after PEG-4000 precipitation of major soluble proteins.

16 kDa polypeptide was in fact the small subunit of Rubisco and also to reveal other low-abundance nitrosylated proteins of *K. pinnata* leaf extracts. The results in Fig. 2B (lane 3) show the absence of the

16 kDa nitrosylated polypeptide from the PEG-treated fraction and three very strongly immunopositive polypeptides (marked with asterisks, lane 3) that were enriched in this fraction.

MALDI-TOF mass spectrometry was used to identify the polypeptides excised from the gel (marked with dots in Fig. 2A), and similarity/identity was ascertained using a Mascot search engine (Matrix Science, London, UK). Table 1 lists the nitrosylated proteins that were identified. The list includes proteins that function in primary and secondary metabolism, photosynthesis, DNA replication, abiotic and biotic stress responses, the cytoskeleton, and a few unknowns. As both subunits of Rubisco appeared to be targets of S-nitrosylation in this study as well as in previous studies on *Arabidopsis* [9], and Rubisco is a key protein in carbon fixation, we investigated it in detail.

### Rubisco small subunit is S-nitrosylated and NO inhibits carbon fixation

The absence of the 16 kDa polypeptide in fractions in which Rubisco protein amounts were decreased, and its identity as the small subunit of Rubisco as revealed by the Mascot search engine (see above), show that it is one of the major targets of nitrosylation in *K. pinnata*. Nitrosylation of the small subunit of Rubisco does not occur in the absence of biotin or the presence of GSH, and is not totally blocked even at higher concentrations of NEM (supplementary Fig. S1).

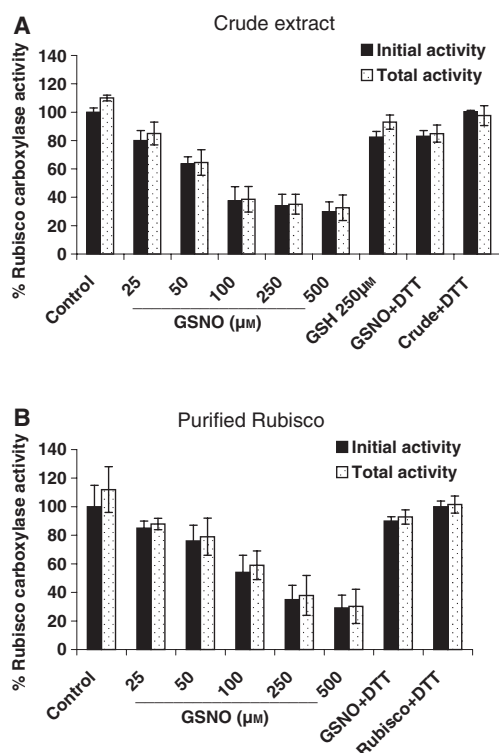
To test the physiological relevance of S-nitrosylation of Rubisco, its activity was analyzed under nitrosylating and non-nitrosylating conditions. Crude extracts of *K. pinnata* were incubated with either GSNO (25–500  $\mu$ M) or GSH (100–500  $\mu$ M) prior to carboxylation assay. Treatment with GSNO reduced both the initial and total carboxylase activity in a dose-dependent manner (Fig. 3A). GSH did not have any significant effect. Addition of 10 mM dithiothreitol to GSNO-treated extract restored the initial and total activities to 83 and 84.9%, respectively. These observations suggest the involvement of thiol group(s) in the nitrosylation of Rubisco. Reactivation of inhibited Rubisco by reducing agents (dithiothreitol, GSH) has been reported previously [11].

To further ascertain whether the GSNO effect is a direct or indirect one, Rubisco was purified according to the method described previously [12], and purified protein (approximately 9  $\mu$ g) was incubated with either GSNO (25–500  $\mu$ M) or GSH (100–500  $\mu$ M) and carboxylase activity determined. Similar to the data obtained with crude extracts, purified Rubisco was inhibited by GSNO in a dose-dependent manner (Fig. 3B). Further,

**Table 1.** Identification of S-nitrosylated proteins from GSNO-treated *Kalanchoe pinnata* leaf. Major polypeptides (marked with dots in Fig. 2A) were excised from the silver-stained gel and subjected to trypsin digestion. Peptide mass was analyzed by MALDI-TOF mass spectrometry. Protein identification was performed using the Mascot search engine, utilizing a probability-based scoring system and the mass spectrometry protein sequence database. Proteins in bold are unique S-nitrosylation targets; italicized proteins are common to those in *Arabidopsis*; underlined are similar to S-nitrosylation targets in animals; rest are common in *Kalanchoe*, *Arabidopsis* and animals. Hypothetical protein, \*not relevant in animals.

Protein identified	Uniprot accession no.	Molecular mass (Da)	Mowse score <sup>a</sup>	Matched peptides	Sequence covered (%)	Reported in Animal systems	<i>A. thaliana</i>	Functional category
<b>Kinesin-like protein</b>	Q9FZ06	100887	74	17	37	N	N	Cytoskeleton protein
Heat shock protein, putative	Q9S7E7	90956	92	14	33	Y	Y	Stress-related proteins
Heat shock protein 81-3	P51818	80052	102	11	35	Y	Y	
<i>Cobalamin-independent methionine synthase</i> , putative	Q9SRV5	84584	107	19	33	N	Y	Sulfur metabolism
Fructose-bisphosphate aldolase (fragment), putative <sup>b</sup>	Q1A7T7	11665	98	1	20	Y	Y	CHO metabolism
Glyceraldehyde 3-phosphate dehydrogenase C subunit	Q8LAS0	36989	74	8	26	Y	Y	CHO metabolism
Phosphoglycerate kinase <sup>b</sup>	Q8LFV7	42148	77	1	4	Y	Y	CHO metabolism
<b>UDP-glucose 4-epimerase, putative</b>	Q8H0B7	39200	68	11	32	N	N	CHO metabolism
Glutamate ammonia ligase <sup>b</sup>	Q56ZK3	19978	70	1	7	Y	N	Nitrogen metabolism
<i>Rubisco large subunit</i>	Q33557	48851	144	20	54	N	Y	Proteins involved in photosynthesis
<i>Rubisco small subunit, precursor<sup>c</sup></i>	Q43746	20191	121	14	69	N	Y	
<i>Rubisco small subunit chain 2B, chloroplast, precursor<sup>c</sup></i>	P10797	20350	70	10	69	N	Y	
Carbonic anhydrase <sup>b</sup>	Q41088	35019	111	1	6	Y	N	
<b>Glycolate oxidase</b>	Q3L1H0	40442	84	10	37	N	N	
Phosphoenolpyruvate carboxylase, isoform 1 (fragment)	Q8VXH1	41080	167	23	73	Y	Y	
<b>DNA topoisomerase II, putative</b>	Q6Z8D9	170335	77	26	32	N	N	Protein involved in DNA replication
Disease resistance protein, putative	Q7XJL6	125883	65	16	49	*	N	Protein involved in disease resistance
Hypothetical protein T17F15:70	Q9SU69	172230	77	21	50	–	–	Unknown
Hypothetical protein At1g26799	Q6AWV3	17343	64	10	33	–	–	

<sup>a</sup> Molecular weight search score (Mowse score). <sup>b</sup> Polypeptides identified using LC-MS/MS. <sup>c</sup> The precursor of the Rubisco small subunit has a molecular mass of 20 kDa but migrates as a 16 kDa protein in SDS-PAGE. Peptide mass fingerprinting of the polypeptide that migrated at 16 kDa in SDS-PAGE showed matches with both the Rubisco small subunit precursor and its mature form (approximately 90% similarity).

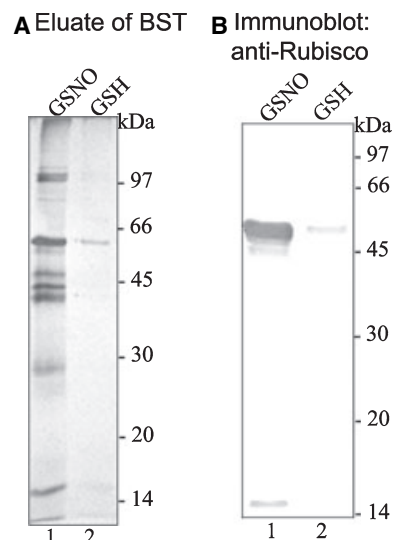


**Fig. 3.** Rubisco activity is inhibited by GSNO. (A) Leaf extracts of *Kalanchoe pinnata* were used either as such (Control) or treated with the indicated concentrations of either GSNO (25–500  $\mu$ M) or GSH (250  $\mu$ M). Enzyme activity was determined as previously described [55]. The initial Rubisco activity in the untreated control extract was taken as 100%. Absolute initial and total activities were 304.8 and 352 nmol CO<sub>2</sub> min<sup>-1</sup> mg<sup>-1</sup> of protein, respectively. Addition of 10 mM dithiothreitol (DTT) to extract inhibited using 250  $\mu$ M GSNO restored the Rubisco activity. (B) Purified Rubisco was used either as such (Control) or first treated with the indicated concentrations of either GSNO (25–500  $\mu$ M) or GSH (250  $\mu$ M) prior to the measurement of enzyme activity. Each treatment consisted of triplicate samples and was repeated three times.

we quantified the nitrosothiol content [13] in the purified Rubisco fraction treated with GSNO (250  $\mu$ M). GSNO-treated lysozyme (with no free thiols) was used as a negative control and did not yield a positive reaction, while GSNO-treated Rubisco protein yielded 41  $\mu$ g of S-nitrosothiols per mg of protein.

### *In vivo* S-nitrosylation of Rubisco

Finally, nitrosylation of Rubisco was analyzed *in vivo*. Leaf discs were incubated with either GSNO (250  $\mu$ M) or GSH (250  $\mu$ M) for 2 h at room temperature in the dark. Leaf extracts were subjected to the biotin switch technique, and biotinylated proteins were purified using neutravidin–agarose as described in Experimental

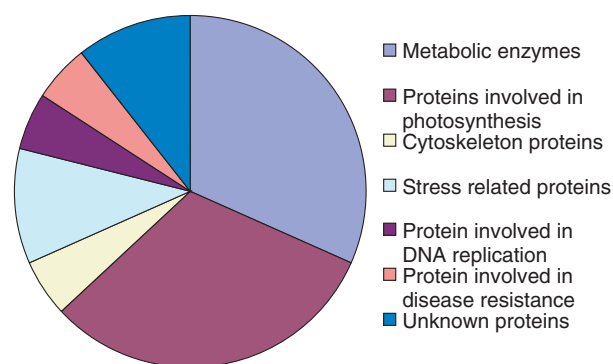


**Fig. 4.** Analysis of *in vivo* S-nitrosylated Rubisco. *Kalanchoe pinnata* leaf discs were incubated with GSNO (250  $\mu$ M) or GSH (250  $\mu$ M) and their extracts were then subjected to the biotin switch technique. (A) Silver-stained SDS–polyacrylamide (12%) gel showing neutravidin–agarose-purified nitrosylated proteins. (B) Immunoblot of purified samples probed using anti-Rubisco IgGs (1 : 1000).

procedures. Eluates were resolved on a gel followed by immunoblotting with anti-Rubisco IgGs. Immunoblot analysis confirmed that both the subunits of Rubisco were nitrosylated *in vivo*, and this nitrosylation was inhibited by GSH (Fig. 4).

### Discussion

We demonstrate that a number of proteins from the medicinal CAM plant *K. pinnata* undergo S-nitrosylation in response to NO-releasing compound. These proteins represent the functional categories DNA replication, cytoskeleton, carbon, nitrogen and sulfur metabolism, plant defense responses and photosynthesis (Table 1 and Fig. 5). Of the identified S-nitrosylated proteins involved in photosynthesis, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was characterized. Its nitrosylation was found to inhibit its activity. This is to our knowledge the first demonstration showing modulation of Rubisco activity by S-nitrosylation. Rubisco plays a central role in photosynthesis. Oxidative stress and thiol-reducing agents are known to target Rubisco and modulate its activity [14–16]. Substitution of a cysteine residue (Cys65) in the Rubisco small subunit induces alterations in the catalytic efficiency and thermal stability of Rubisco [17]. Based on these data, Rubisco may be predicted to be a potential S-nitrosylation target. Our results



**Fig. 5.** Functional categories of *Kalanchoe pinnata* S-nitrosylated proteins. The identified S-nitrosylated proteins were classified into various functional categories as shown. The area for each category indicates the relative percentage of proteins in that category.

showing a linkage between post-translational S-nitrosylation of Rubisco and its enzymatic activity suggest that NO can have an impact on photosynthesis. Reports of NO-mediated inhibition of photosynthesis have been published previously [18,19], but the mechanism was not known until now. NO is known to be generated in the chloroplasts [20], and it was suggested that reactive nitrogen species could exert an effect on chloroplast macromolecules [20–22]. Our data support this assertion, and identify a number of chloroplast soluble proteins in addition to Rubisco as targets of NO action via S-nitrosylation.

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31), carbonic anhydrase (EC 4.2.1.1) and glycolate oxidase (EC 1.1.3.15) feature among the list of identified S-nitrosylated proteins of *K. pinnata*. PEPC is an important enzyme that catalyzes the primary step in fixing atmospheric CO<sub>2</sub> in C<sub>4</sub> and CAM plants, generating oxaloacetate from phosphoenolpyruvate. In C<sub>4</sub> plants, PEPC is regulated by light [23], and in CAM plants it is regulated by reversible phosphorylation, involving PEPC kinase, which is under the control of a circadian clock and phosphorylates PEPC in the dark [23,24]. However, post-translational modification of PEPC by nitrosylation occurs in both *Arabidopsis*, a C<sub>3</sub> plant [9], and *K. pinnata*, a CAM plant (this study).

Carbonic anhydrase is present in animals, plants, eubacteria and viruses [25]. S-glutathiolation of mammalian carbonic anhydrase III protein sulfhydryl groups has been shown previously [26]. In *Arabidopsis*, neither carbonic anhydrase nor glycolate oxidase were found among the nitrosylated proteins [9]. Flavin mononucleotide-dependent glycolate oxidase catalyzes the oxidation of  $\alpha$ -hydroxy acids to the corresponding  $\alpha$ -ketoacids, and is one of the green plant enzymes

involved in photorespiration. Nitrogen status influences the structure and activity of this enzyme in an aquatic angiosperm [27]. In animals, the enzyme participates in the production of oxalate [28].

A number of proteins associated with carbohydrate, nitrogen and sulfur metabolism are among the identified *K. pinnata* S-nitrosylated proteins. Those involved in carbohydrate metabolism include fructose-1,6-bisphosphate aldolase, the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) C subunit, phosphoglycerate kinase and UDP glucose 4-epimerase. All these enzymes except putative UDP glucose 4-epimerase were previously identified as S-nitrosylated targets in *Arabidopsis* [9]. Like carbonic anhydrase, both aldolase and phosphoglycerate kinase are glutathionylated under oxidative stress in human T lymphocytes [29]. Evidence for S-glutathionylation of GAPDH by NO has been presented previously [30]. The above described characteristics are consistent with these proteins to be S-nitrosylated. Thus, glycolysis and galactose metabolic components are also the targets of NO.

Glutamate ammonia ligase (or glutamine synthetase; EC 6.3.1.2) plays a central role in nitrogen metabolism by catalyzing the synthesis of glutamine from glutamate, ATP and ammonium [31]. Despite being a key enzyme in nitrogen metabolism, little is known about the regulatory mechanisms controlling plant glutamine synthetase at the post-translational level. Oxidative stress targets soybean root glutamine synthetase for proteolysis *in vitro*, and exogenous application of ammonium nitrate induces the glutamine synthetase transcript and protein [32]. Surprisingly, glutamine synthetase in soybean supplemented with exogenous nitrogen is less susceptible to oxidative modification and proteolysis. In isolated pea chloroplasts, light was shown to cause degradation of soluble proteins, including glutamine synthetase [33].

The plant cobalamin-independent methionine synthase (EC 2.1.1.14) is an important enzyme that synthesizes methionine, which is linked to two metabolic networks, sulfur and carbon metabolism [34]. The finding that enzymes in carbon, nitrogen and sulfur metabolism are targets of S-nitrosylation may have important implications in the regulation of carbon, nitrogen and sulfur fluxes in plants under normal as well as stress conditions. Future studies and further characterization should provide information regarding those effects. However, the fact that the chaperone proteins, high-molecular-weight heat-shock proteins (HSP) 90 and 81.3, are among the nitrosylated proteins in *Arabidopsis* (HSP90) [9] and *K. pinnata* (HSP90 and HSP81.3) is an indication of important implications for cellular metabolism following sensing of NO.

Over 120 proteins are known to be S-nitrosylated in animal systems [35–43]. However, information on protein nitrosylation in plants is limited. The only other plant in which S-nitrosylated proteins have been identified is *Arabidopsis* [9]. A comparison of the identified *K. pinnata* S-nitrosylated proteins with those in *Arabidopsis* reveals common targets as heat shock proteins, fructose-1,6-bisphosphate aldolase, and the large and small subunits of Rubisco. S-nitrosylated proteins identified here that have not been identified previously include putative UDP-glucose 4-epimerase, glycolate oxidase, kinesin-like protein, putative DNA topoisomerase II and a putative disease resistance protein that shows homology to cytoplasmic nucleotide-binding site/leucine-rich repeat (NBS-LRR) proteins (Table 1). Thus, studies on the CAM plant *K. pinnata* indicate that other proteins are S-nitrosylated, in addition to the NO-modified proteins shared with the C<sub>3</sub> plant, *Arabidopsis*. All of these S-nitrosylated proteins have cysteine residues. Future studies are required to address whether S-nitrosylation alters their activity and which cysteine(s) is the most likely target(s) for S-nitrosylation.

Kinesin-like protein, putative DNA topoisomerase and the putative disease resistance protein identified here among the S-nitrosylated proteins have not featured in previous reports with animal systems [35–43] or *Arabidopsis* [9]. However, other cytoskeleton proteins such as actin and tubulin were shown to undergo S-nitrosylation in *Arabidopsis* [9]. Kinesins are eukaryotic microtubule-associated motor proteins that have roles in vesicle and organelle transport, cell movement, spindle formation and chromosome movement [44]. DNA topoisomerase II, an enzyme that removes DNA supercoiling by catalyzing DNA swiveling and relaxation and that affects macromolecular biosynthesis, is also S-nitrosylated [45,46].

The presence of a putative nucleotide-binding site/leucine-rich repeat (NBS-LRR)-type disease resistance protein among the identified S-nitrosylated proteins in *K. pinnata* is interesting and consistent with previous findings that S-nitrosothiols play a central role in plant disease resistance [47]. NO levels have been associated with signaling in plant disease resistance [48]. Our data suggests that NO signaling in plant disease resistance may involve nitrosylation of disease resistance proteins.

In conclusion, given that S-nitrosylation encompasses kinesins that function in cell division and development processes, DNA topoisomerase II that functions in the transcription and replication of DNA, enzymes involved in carbon, nitrogen and sulfur metabolism, proteins involved in photosynthesis and photorespiration, defense-related proteins and several

unknowns, NO-mediated protein S-nitrosylation is likely to have broader implications in plant processes than realized so far. The identification of 19 S-nitrosylated proteins in *K. pinnata* was carried out by *in vitro* treatment with GSNO, which is commonly used as a source of NO generation to study NO effects. The *in vivo* concentrations of GSNO in *K. pinnata* are not known. However, similar protein targets were identified at the concentrations of GSNO (in  $\mu\text{M}$  range) used here, and *in vitro* with GSNO and *in vivo* with NO gas in *Arabidopsis*. Therefore, it is likely that the same protein targets are S-nitrosylated *in vivo*. Although we have presented detailed studies on the nitrosylation of Rubisco and inhibition of this major enzyme, *in vivo* validation of the identified protein targets is required. It will also be important to monitor NO levels *in planta* in response to developmental and environmental cues. Recently, it was observed that S-nitrosothiol levels increase in response to abiotic stress in olive seedlings [47]. In addition, S-nitrosothiols and NO have been shown to play role in biotic stress [48,49].

When studying NO signaling and its components, it is critical to elucidate the S-nitrosoproteome of not only model plants but also of cash crops, as profiling of the S-nitrosoproteome in response to environmental and developmental cues has the potential to provide novel targets for crop improvement.

## Experimental procedures

### Materials

GSNO, GSH, neocuproine, sodium ascorbate, Hepes, Triton X-100, ribulose-1,5-bisphosphate and anti-biotin mouse monoclonal IgG were obtained from Sigma-Aldrich (St Louis, MO, USA). Methyl methanethiosulfonate (MMTS), NEM, *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide (biotin-HPDP) and neutravidin-agarose were obtained from Pierce (Rockford, IL, USA). Anti-mouse IgG alkaline phosphatase conjugate was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).  $\text{NaH}^{14}\text{CO}_3$  was obtained from the Board of Radiation and Isotope Technology (Mumbai, India). Teepol (neutral liquid detergent) was purchased from Reckitt Benckiser (Haryana, India). All other chemicals used were of analytical grade.

### Plant material and growth conditions

*Kalanchoe pinnata* plants were grown in the botanical garden at the University of Delhi, India. The third pair of leaves from the apex was excised and used for the

experiments. Leaves were surface-sterilized in 1% Teepol, thoroughly rinsed with sterile distilled water, and then dried in a laminar flow hood.

### Protein extraction and PEG-4000 precipitation

Frozen leaf discs were extracted (1 : 3 w/v) in TEGN buffer (500 mM Tris/HCl pH 8.0, 5 mM EDTA, 15% glycerol and 0.1 mM neocuproine), and the extracts were centrifuged at 14 000 *g* for 30 min (at 4 °C) to remove the particulates. Supernatants were used for protein estimation using the Bradford assay [50] with BSA as the standard. To remove the major leaf protein, Rubisco, from the extracts, 60% w/v PEG-4000 was added to the supernatant (final concentration 15% PEG-4000) with stirring. After 30 min of stirring at 4 °C, the solution was centrifuged at 16 700 *g* for 45 min (at 4 °C). The supernatant was retained for analysis.

### Detection of S-nitrosylated proteins by the biotin-switch technique

S-nitrosylated proteins were detected using the biotin switch technique [51]. Briefly, the protein concentration in the supernatant was adjusted to 0.8 µg·µL<sup>-1</sup> using HEN solution (25 mM Hepes/NaOH pH 7.7, 1 mM EDTA, 0.1 mM neocuproine) and incubated with GSNO or GSH for 20 min at room temperature. Proteins were acetone-precipitated to remove GSNO or GSH, and then incubated at 50 °C for 20 min in 50 mM NEM and 2.5% SDS (prepared in HEN solution) with frequent vortexing. Another acetone precipitation was performed to remove NEM. The protein pellet was re-suspended in 0.1 mL HENS solution (HEN solution in 1% SDS) per mg protein, followed by incubation with 2 mM biotin-HPDP and 5 mM ascorbate for 1 h at 25 °C. Assay components were optimized for *K. pinnata*. Leaf extracts (240 µg protein) treated with 0, 100, 250, 500 or 700 µM GSNO for 20 min showed the same S-nitrosylation pattern but increased intensity (Fig. 1). Based on these results, GSNO (250 µM) was used for the remaining experiments to avoid secondary reactions such as production of free S-nitrosothiols or NO<sub>2</sub> formation [42]. The assay was also performed without blocking the proteins (with MMTS or NEM) as a positive control (Fig. 1, no block). Free thiol blockage by treatment with NEM was tested at 50–500 mM and at various temperatures (45–55 °C). NEM at or above 50 mM completely blocked free thiols in all S-nitrosylated proteins, with a reduced effect on the 16 kDa polypeptide (supplementary Fig. S1). Varying the incubation temperature (45–55 °C) did not alter the profile; therefore 50 °C was used as the blocking temperature [35]. Another reversible sulfonating reagent, MMTS, gave similar results when used as a blocking agent at 20 mM. The experiments presented were repeated at least three times.

### Purification and identification of S-nitrosylated proteins

Biotinylated proteins were precipitated, washed with pre-chilled acetone and re-suspended in HENS solution (0.1 mL·mg<sup>-1</sup> protein). Two volumes of neutralization buffer (20 mM Hepes/NaOH pH 7.7, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) were added. Neutravidin-agarose at 15 µL·mg<sup>-1</sup> of protein was added, and the mixture was incubated for 1 h at room temperature. The resin was washed six times with ten volumes of washing buffer (neutralization buffer with 600 mM NaCl). Elution was carried out with 400 µL elution buffer (neutralization buffer containing 100 mM β-mercaptoethanol) for 20 min. After acetone precipitation, the pellet was dissolved in HENS and SDS sample buffer (reducing). Proteins were resolved by 12% SDS-PAGE [52] and visualized by silver staining [53]. The S-nitrosylated protein purification procedure was repeated three times.

Protein bands were excised from the gel, digested with trypsin and identified either by peptide mass fingerprinting; MALDI-TOF MS (Bruker Daltonics, Billerica, MA, USA) or LC-MS/MS at the Centre for Genomic Application, New Delhi (India). Each set of peptides obtained was matched using the Mascot search engine (Matrix Science), utilizing a probability-based scoring system and a mass spectrometry protein database. Those matches found to be significant using the Mascot search engine algorithm were classed as identified. The Mascot scoring system calculates the random event probability of matches between the experimental data and mass values (calculated from a candidate peptide or protein sequence) using the equation  $-10 \log_{10}(P)$ , where *P* is the probability. If the probability is high, it is taken as a false-positive, while a true match would have a low probability value. The mass spectrometry protein sequence database is a composite, non-identical protein sequence database, built from a number of primary source databases such as PIR, TrEMBL, GenBank, Swiss-Prot and NRL3D.

### Immunoblotting

Biotinylated proteins were mixed with SDS sample buffer without reducing agents, separated by 12% SDS-PAGE, and transferred onto nitrocellulose membrane using a semi-dry apparatus (GE Healthcare, Uppsala, Sweden). Immunoblotting was performed as described previously [54]. Immunoblots were blocked with 3% BSA and then probed with either anti-biotin mouse monoclonal IgG (Sigma) at a dilution of 1 : 500 or anti-Rubisco IgG for 2 h at a dilution of 1 : 1000. Alkaline phosphatase-conjugated antibodies (Santa Cruz) were added, and cross-reacting protein bands were visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

### Rubisco carboxylase activity assay

Leaf discs (600 mg fresh weight) were extracted in 2 mL extraction buffer (250 mM Bicine pH 8.0, 10 mM MgCl<sub>2</sub>, 5 mM EDTA, 2% w/v PVP, 15% w/v PEG-20 000, 2.5% v/v Tween-20, 1 mM phenylmethanesulfonyl fluoride) and centrifuged at 10 000 *g* for 30 s at 4 °C. The supernatant was incubated with and without GSNO (250 µM) or GSH (250 µM) for 20 min at room temperature in the dark. Rubisco activity can be modulated through reversible carbamylation in response to change in light intensity, CO<sub>2</sub> or O<sub>2</sub>. In order to discount this, Rubisco activity was measured [55] as soon as the extracts were prepared (initial activity) and after incubating them with saturating concentrations of CO<sub>2</sub> and Mg<sup>2+</sup> to carbamylate Rubisco (total activity) [56]. Briefly, for initial Rubisco activity, 100 µL of each sample was added to 400 µL of assay buffer (166 mM Bicine/KOH pH 8.0, 10 mM MgCl<sub>2</sub>, 30 µM NaH<sup>14</sup>CO<sub>3</sub> at 51 Ci·mol<sup>-1</sup>). The reaction was initiated by addition of the substrate ribulose-1,5-bisphosphate (0.5 mM) and terminated after 1 min using 200 µL of 5 N HCl. Total activity was measured by pre-incubating each sample for 8 min at 30 °C prior to the addition of ribulose-1,5-bisphosphate. After terminating the reaction, the samples were dried overnight and the acid-stable <sup>14</sup>C counts were determined using a liquid scintillation counter. To reactivate the enzyme, GSNO-inhibited enzyme was treated with 10 mM dithiothreitol for 20 min at room temperature, residual dithiothreitol was removed by gel filtration, and the protein was assayed for Rubisco activity as described above. Each experiment was carried out in triplicate and repeated three times.

Rubisco holoenzyme from *K. pinnata* was purified by the method described previously [12]. The purified protein was treated with either GSNO (250 µM) or GSH (250 µM) for 20 min at room temperature in the dark. The initial and total Rubisco activities were then determined as described above.

### In vivo S-nitrosylation of Rubisco

*K. pinnata* leaf discs were incubated with either GSNO (250 µM) or GSH (250 µM) for 2 h at room temperature in the dark. Soluble proteins were isolated and subjected to the biotin switch technique as described above. Biotinylated proteins were purified, resolved by SDS-PAGE, and immunoblotted with anti-Rubisco IgG as described above. Each treatment was repeated three times.

### Quantification of S-nitrosothiols

S-nitrosothiols were quantified as described previously [13]. Briefly, 180 µL of purified Rubisco protein (34 µg protein equivalent) was either treated or not treated with 250 µM GSNO. After removing residual GSNO by acetone precipitation, the pellets were dissolved in 180 µL HEN solution. To this, 30 µL of 0.5% ammonium sulfamate was added.

After 2 min incubation, the solution was made to 2.7% sulfanilamide and 0.25% HgCl<sub>2</sub> (in 0.4 N HCl) in a final volume of 300 µL. Finally, 240 µL of freshly prepared 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride was added. After 20 min incubation at room temperature, the absorbance was measured at 540 nm. S-nitrosothiol content was determined using a standard curve prepared with different concentrations of GSNO.

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### References

- 1 Lamattina L, García-Mata C, Graziano M & Pagnussat G (2003) Nitric oxide: the versatility of an extensive signal molecule. *Annu Rev Plant Biol* **54**, 109–136.
- 2 Neill SJ, Desikan R & Hancock JT (2003) Nitric oxide signalling in plants. *New Phytol* **159**, 11–35.
- 3 Delledonne M (2005) NO news is good news for plants. *Curr Opin Plant Biol* **8**, 390–396.
- 4 Gow AJ, Farkouh CR, Munson DA, Posencheg MA & Ischiropoulos H (2004) Biological significance of nitric oxide-mediated protein modifications. *Am J Physiol* **287**, L262–L268.
- 5 Broillet MC (1999) S-nitrosylation of proteins. *Cell Mol Life Sci* **55**, 1036–1042.
- 6 Mannick JB & Schonhoff CM (2002) Nitrosylation: the next phosphorylation? *Arch Biochem Biophys* **408**, 1–6.
- 7 Taldone FS, Tummala M, Goldstein EJ, Ryzhov V, Ravi K & Black SM (2005) Studying the S-nitrosylation of model peptides and eNOS protein by mass spectrometry. *Nitric Oxide* **13**, 176–187.
- 8 Parani M, Rudrabhatla S, Myers R, Weirich H, Smith B, Leaman DW & Goldman SL (2004) Microarray analysis of nitric oxide responsive transcripts in *Arabidopsis*. *Plant Biotechnol J* **2**, 359–366.
- 9 Lindermayr C, Saalbach G & Durner J (2005) Proteomic identification of S-nitrosylated proteins in *Arabidopsis*. *Plant Physiol* **137**, 921–930.
- 10 Taylor L (2004) *The Healing Power of Rainforest Herbs*. Square One Publishers Inc., Garden City Park, NY.

- 11 Gracia-Ferris C & Moreno J (1993) Redox regulation of enzymatic activity and proteolytic susceptibility of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Euglena gracilis*. *Photosynth Res* **35**, 55–66.
- 12 Hall NP & Tolbert NE (1978) A rapid procedure for the isolation of ribulose bisphosphate carboxylase/oxygenase from spinach leaves. *FEBS Lett* **96**, 167–169.
- 13 Saville B (1958) A scheme for the colorimetric determination of microgram amounts of thiols. *Analyst* **83**, 670–672.
- 14 Mehta RA, Fawcett TW, Porath D & Mattoo AK (1992) Oxidative stress causes rapid membrane translocation and *in vivo* degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase. *J Biol Chem* **267**, 2810–2816.
- 15 Desimone M, Henke A & Wagner E (1996) Oxidative stress induces partial degradation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in isolated chloroplasts of barley. *Plant Physiol* **111**, 789–796.
- 16 Marcus Y, Altman-Gueta H, Finkler A & Gurevitz M (2003) Dual role of cysteine 172 in redox regulation of ribulose 1,5-bisphosphate carboxylase/oxygenase activity and degradation. *J Bacteriol* **185**, 1509–1517.
- 17 Genkov T, Du YC & Spreitzer RJ (2006) Small-subunit cysteine-65 substitutions can suppress or induce alterations in the large-subunit catalytic efficiency and holoenzyme thermal stability of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Arch Biochem Biophys* **451**, 167–174.
- 18 Bruggink GT, Wolting HG, Dassen JHA & Bus VGM (1988) The effect of nitric oxide fumigation at two CO<sub>2</sub> concentrations on net photosynthesis and stomatal resistance of tomato (*Lycopersicon lycopersicum* L. cv. Abunda). *New Phytol* **110**, 185–191.
- 19 Caporn SJM, Hand DW, Mansfield TA & Wellburn AR (1994) Canopy photosynthesis of CO<sub>2</sub>-enriched lettuce (*Lactuca sativa* L.). Response to short-term changes in CO<sub>2</sub>, temperature and oxides of nitrogen. *New Phytol* **126**, 45–52.
- 20 Jasid S, Simontacchi M, Bartoli CG & Puntarulo S (2006) Chloroplasts as a nitric oxide cellular source. Effect of reactive nitrogen species on chloroplastic lipids and proteins. *Plant Physiol* **142**, 1246–1255.
- 21 Foyer CH & Allen JF (2003) Lessons from redox signaling in plants. *Antioxid Redox Signal* **5**, 3–5.
- 22 Grun S, Lindermayr C, Sell S & Durner J (2006) Nitric oxide and gene regulation in plants. *J Exp Bot* **57**, 507–516.
- 23 Hartwell J, Gill A, Nimmo GA, Wilkins MB, Jenkins GI & Nimmo HG (1999) Phosphoenolpyruvate carboxylase kinase is a novel protein kinase regulated at the level of expression. *Plant J* **20**, 333–342.
- 24 Taybi T, Patil S, Chollet R & Cushman JC (2000) A minimal serine/threonine protein kinase circadianly regulates phosphoenolpyruvate carboxylase activity in crassulacean acid metabolism-induced leaves of the common ice plant. *Plant Physiol* **123**, 1471–1481.
- 25 Lazova GN, Naidenova T & Velinova K (2004) Carbonic anhydrase activity and photosynthetic rate in the tree species *Paulownia tomentosa* Steud. Effect of dimethylsulfoxide treatment and zinc accumulation in leaves. *J Plant Physiol* **161**, 295–301.
- 26 Ji Y, Akerboom TPM, Sies H & Thomas JA (1999) S-nitrosylation and S-glutathiolation of protein sulfhydryls by S-nitroso glutathione. *Arch Biochem Biophys* **362**, 67–78.
- 27 Emes MJ & Erismann KH (1982) The influence of the nitrogen supply on the structure and activity of glycolate oxidase in *Lemna minor* L. *Plant Sci Lett* **27**, 103–109.
- 28 Stenberg K & Lindqvist Y (1997) Three dimensional structures of glycolate oxidase with bound active-site inhibitors. *Protein Sci* **6**, 1009–1015.
- 29 Fratelli M, Demol H, Puype M, Casagrande S, Eberini I, Salmona M, Bonetto V, Mengozzi M, Duffieux F, Miclet E *et al.* (2002) Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc Natl Acad Sci USA* **99**, 3505–3510.
- 30 Mohr S, Hallak H, Boitte A, Lapetina EG & Brüne B (1999) Nitric oxide induced S-glutathionylation and inactivation of glyceraldehyde 3-phosphate dehydrogenase. *J Biol Chem* **274**, 9427–9430.
- 31 Lima L, Seabra A, Melo P, Cullimore J & Carvalho H (2006) Post-translational regulation of cytosolic glutamine synthetase of *Medicago truncatula*. *J Exp Bot* **57**, 2751–2761.
- 32 Ortega JL, Roche D & Sengupta-Gopalan C (1999) Oxidative turnover of soybean root glutamine synthetase *in vitro* and *in vivo* studies. *Plant Physiol* **119**, 1483–1495.
- 33 Stieger PA & Feller U (1997) Requirements for the light-stimulated degradation of stromal proteins in isolated pea (*Pisum sativum* L.) chloroplasts. *J Exp Bot* **48**, 1639–1645.
- 34 Ravel S, Block MA, Rippert P, Jabrin S, Curien G, Rebeille F & Douce R (2004) Methionine metabolism in plants. Chloroplasts are autonomous for de novo methionine synthesis and can import S-adenosylmethionine from the cytosol. *J Biol Chem* **279**, 22548–22557.
- 35 Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P & Snyder SH (2001) Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat Cell Biol* **3**, 193–197.
- 36 Kunciewicz T, Sheta EA, Goldknopf IL & Kone BC (2003) Proteomic analysis of S-nitrosylated proteins in mesangial cells. *Mol Cell Proteomics* **2**, 156–163.
- 37 Foster MW & Stamler JS (2004) New insights into protein S-nitrosylation. Mitochondria as a model system. *J Biol Chem* **279**, 25891–25897.

- 38 Gao C, Guo H, Wei J, Mi Z, Wai PY & Kuo PC (2005) Identification of S-nitrosylated proteins in endotoxin-stimulated RAW264.7 murine macrophages. *Nitric Oxide* **12**, 121–126.
- 39 Rhee KY, Erdjument-Bromage H, Tempst P & Nathan CF (2005) S-nitrosoproteome of *Mycobacterium tuberculosis*: enzymes of intermediary metabolism and antioxidant defense. *Proc Natl Acad Sci USA* **102**, 467–472.
- 40 Yang Y & Loscalzo J (2005) S-nitrosoprotein formation and localization in endothelial cells. *Proc Natl Acad Sci USA* **102**, 117–122.
- 41 Zhang Y, Keszler A, Broniowska KA & Hogg N (2005) Characterization and application of the biotin-switch assay for the identification of S-nitrosated proteins. *Free Radic Biol Med* **38**, 874–881.
- 42 Burwell LS, Nadtochiy SM, Tompkins AJ, Young S & Brookes PS (2006) Direct evidence for S-nitrosation of mitochondrial complex I. *Biochem J* **394**, 627–634.
- 43 Hao G, Derakhshan B, Shi L, Campagne F & Gross SS (2006) SNOSID, a proteomic method for identification of cysteine S-nitrosylation sites in complex protein mixtures. *Proc Natl Acad Sci USA* **103**, 1012–1017.
- 44 Reddy AS (2001) Molecular motors and their functions in plants. *Int Rev Cytol* **204**, 97–178.
- 45 Xie S & Lam E (1994) Abundance of nuclear DNA topoisomerase II is correlated with proliferation in *Arabidopsis thaliana*. *Nucleic Acids Res* **22**, 5729–5736.
- 46 Singh BN, Sopory SK & Reddy MK (2004) Plant DNA topoisomerases: structure, function, and cellular roles in plant development. *Crit Rev Plant Sci* **23**, 251–269.
- 47 Feechan A, Kwon E, Yun BW, Wang Y, Pallas JA & Loake GJ (2005) A central role for S-nitrosothiols in plant disease resistance. *Proc Natl Acad Sci USA* **102**, 8054–8059.
- 48 Delledonne M, Xia Y, Dixon RA & Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. *Nature* **394**, 585–588.
- 49 Valderrama R, Corpas FJ, Fernandez-Ocaña A, Chaki M, Luque F, Gómez-Rodríguez MV, Colmenero-Varea P, Río LA & Barroso JB (2007) Nitrosative stress in plants. *FEBS Lett* **581**, 453–461.
- 50 Bradford MM (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.
- 51 Jaffrey SR & Snyder SH (2001) The biotin switch assay for the detection of S-nitrosylated proteins. *Sci STKE* *PL1*, doi:10.1126/stke.2001.86.p11.
- 52 Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- 53 Merrill CR, Goldman D & Vankeuren ML (1983) Silver staining methods for polyacrylamide gel electrophoresis. *Methods Enzymol* **96**, 230–239.
- 54 Towbin H, Staehelin T & Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**, 4350–4354.
- 55 Maxwell K, Borland AM, Haslam RP, Helliker BR, Roberts A & Griffiths H (1999) Modulation of Rubisco activity during the diurnal phases of the crassulacean acid metabolism plant *Kalanchoë daigremontiana*. *Plant Physiol* **121**, 849–856.
- 56 Parry MAJ, Andralojc PJ, Parmar S, Keys AJ, Habash D, Paul MJ, Alred R, Quick WP & Servaites JC (1997) Regulation of Rubisco by inhibitors in the light. *Plant Cell Environ* **20**, 528–534.

## Supplementary material

The following supplementary material is available online:

**Fig. S1.** Effect of NEM, absence of biotin and presence of dithiothreitol on protein blocking prior to biotinylation.

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